

Insights into dietary flavonoids as molecular templates for the design of anti-platelet drugs

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Received 29 June 2012; revised 19 September 2012; accepted 24 September 2012; online publish-ahead-of-print 27 September 2012

Abstract

Flavonoids are low-molecular weight, aromatic compounds derived from fruits, vegetables, and other plant components. The consumption of these phytochemicals has been reported to be associated with reduced cardiovascular disease (CVD) risk, attributed to their anti-inflammatory, anti-proliferative, and anti-thrombotic actions. Flavonoids exert these effects by a number of mechanisms which include attenuation of kinase activity mediated at the cell-receptor level and/or within cells, and are characterized as broad-spectrum kinase inhibitors. Therefore, flavonoid therapy for CVD is potentially complex; the use of these compounds as molecular templates for the design of selective and potent small-molecule inhibitors may be a simpler approach to treat this condition. Flavonoids as templates for drug design are, however, poorly exploited despite the development of analogues based on the flavanol, isoflavone, and isoflavanone subgroups. Further exploitation of this family of compounds is warranted due to a structural diversity that presents great scope for creating novel kinase inhibitors. The use of computational methodologies to define the flavonoid pharmacophore together with biological investigations of their effects on kinase activity, in appropriate cellular systems, is the current approach to characterize key structural features that will inform drug design. This focussed review highlights the potential of flavonoids to guide the design of clinically safer, more selective, and potent small-molecule inhibitors of cell signalling, applicable to anti-platelet therapy.

Keywords

Flavonoids • Anti-platelet drugs • Drug design • Cardiovascular disease • Flavonoid analogues

1. Dietary flavonoids as negative modulators of cardiovascular disease risk

1.1 Epidemiology and clinical trials

The flavonoid group of compounds comprises flavanol and flavonol,^{1–4} flavan-3-ol, flavan-4-ol, and flavan-3,4-diol subgroups,^{5–7} flavanone,^{8,9} flavone,^{1,2} isoflavone,^{10,11} as well as anthocyanidin and proanthocyanidin^{8,12} subgroups.¹ The potential value of flavonoids as therapy for cardiovascular diseases (CVD) was recognized with the correlation of the consumption of dietary sources containing high levels of these compounds to lowered risk for these conditions.^{1,3,13} A number of epidemiological studies described an association between reduction in the incidence of myocardial infarction (MI) and stroke^{3,13} and dietary intake of flavonoids.

Key studies included a 5-year follow-up study involving 805 men aged 65–84; 38 men from a group of 693 with no history of MI showed an inverse link between flavonoid intake (25 mg/day) and mortality from coronary heart disease.³ Another study demonstrated

that lowered incidence of stroke within a cohort of 552 men aged 50–69 correlated with high-dietary intake of flavonoids.¹³ More recent studies with significantly larger cohorts (1000–90 000 participants), and follow-up periods of up to 11 years, have correlated flavonoid intake with lowered risk of death from CVD,^{14–18} a protective effect against peripheral arterial occlusive disease¹⁹ and prevention of coronary heart disease.²⁰ There are, however, inconsistencies in the evidence base, with some epidemiological studies reporting no association between the dietary intake of flavonols (e.g. quercetin) and ischaemic heart disease²¹ or plasma antioxidant levels.²²

Clinical trials have demonstrated the effects of dietary sources of flavonoids on CVD risk factors,^{23–25} post-thrombotic syndrome,²⁵ atherosclerosis,²⁴ and vascular health^{26–29} (Table 1). A meta-analysis of randomized trials describing the effects of chocolate and cocoa consumption on CVD risk showed that flow-mediated dilatation of blood vessels improved after chronic and acute intakes of these flavan-3-ol-rich food sources.²³ The flavonoid supplement, Pycnogenol® was significantly more effective than compression stockings for relieving oedema symptoms associated with post-thrombotic

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Table 1 Flavonoid clinical trials

Flavonoid	Clinical trial	Outcome of clinical trial	Reference
Flavan-3-ols (chocolate, cocoa, and epicatechin)	Medline, EMBASE, and Cochrane databases for randomized controlled trials of chocolate, cocoa, or flavan-3-ols were reviewed. Forty-two acute or short-term chronic that comprised 1297 participants were assessed.	Insulin resistance was improved by chocolate or cocoa due to significant reductions in serum insulin. Chocolate or cocoa improved flow-mediated dilatation regardless of the dose consumed and reduced diastolic blood pressure, but doses of >50 mg epicatechin/day were needed to induce greater effects on systolic and diastolic blood pressure.	Hooper et al. ²³
High-flavonoid diet	The Zutphen Elderly Study: A high flavonoid diet [tea (61%), onions (13%), and apples (10%)] was given to 805 men aged 65–84. Flavonoid intake was 25.9 mg daily. The men were then followed-up for 5 years.	Between 1985 and 1990, 43 men died of coronary heart disease. Fatal or non-fatal MI occurred in 38 of 693 men with no history of MI at baseline. Flavonoid intake was significantly inversely associated with mortality from coronary heart disease (P for trend = 0.015).	Hertog et al. ³
Pycnogenol [®] (flavonoid supplement from pine bark)	One hundred and fifty-six patients with a single, major episode of proximal deep vein thrombosis were assigned to one of three groups receiving treatment with either compression stockings (group 1), Pycnogenol [®] (group 2), or the combination of both (group 3) for 1 year.	Pycnogenol [®] was significantly more effective from 6 months onwards than compression stockings for relieving oedema symptoms ($P < 0.05$). Symptoms were more effectively reduced with the combination of Pycnogenol [®] and compression stockings than with the individual regimen alone ($P < 0.05$).	Errichi et al. ²⁶
Cranberry juice	An acute pilot study with no placebo ($n = 15$) and a chronic placebo-controlled crossover study ($n = 44$) that examined the effects of cranberry juice on vascular function in subjects with coronary artery disease, for 4 weeks with a 2-week washout period between juice and placebo.	Improved brachial artery flow-mediated dilation was observed 4 h after consumption of a single 480 mL portion of cranberry juice. Chronic (54% juice, 835 mg total polyphenols, and 94 mg anthocyanins) cranberry juice consumption reduced carotid femoral pulse wave velocity.	Dohadwala et al. ²⁷
Grape seeds	In a double-blind, randomized, placebo-controlled intervention study, 28 male smokers were supplemented with 200 mg per day of monomeric and oligomeric flavanols (MOF) from grape seeds. Biomarkers for major pathological processes occurring in the vasculature: disturbances in lipid metabolism and cellular redox balance, and activation of inflammatory cells and platelets were monitored.	In the MOF group, serum total cholesterol and LDL decreased significantly ($P \leq 0.05$). MOF supplementation exerted anti-inflammatory effects in blood towards <i>ex vivo</i> added bacterial endotoxin and significantly reduced the expression of inflammatory genes in leucocytes. A global, vascular health index (of all measured effects) showed a significant improvement of overall vascular health by MOF compared with placebo ($P \leq 0.05$).	Weseler et al. ²⁸
Soya isoflavones	The combined effect of exercise and isoflavones in overweight-to-obese post-menopausal women with clinical risk factors for CVD was assessed over 6 months. The supplementation contained 70 mg/day of isoflavones.	Isoflavones improved leg fat mass % ($P = 0.05$), but interactions were not observed between isoflavones and exercise.	Choquette et al. ²⁹

A number of early and recent human clinical trials demonstrate that flavonoid-rich food sources (e.g. chocolate, red wine, and cranberry juice) reduce CVD risk, by improving vascular health and modulating risk factors linked to associated conditions including thrombosis and atherosclerosis.

syndrome.²⁶ The phenolic content of red wine was suggested to modulate leucocyte adhesion molecules, a marker of atherosclerosis, in patients at high risk from CVD.²⁴ Moreover, chronic cranberry juice (containing high levels of anthocyanins) consumption reduced carotid femoral pulse wave velocity, a clinically relevant measure of arterial stiffness, in subjects with coronary artery disease.²⁷

These data are consistent with flavonoid bioavailability profiles which indicate that these compounds have access to the cells in the circulation (including platelets) at concentrations potentially capable of eliciting modulatory effects. Flavanones (naringenin), flavones (apigenin), flavonols (quercetin), and flavan-3-ols (epicatechin) were detected in the plasma at 5.99,³⁰ 0.1,³¹ 10.66,³² and 5.9 μM ,³³ respectively, after ingestion of supplements and dietary sources. Moreover, elimination of flavonoid metabolites from plasma varies depending on the subclass of compound with half-lives (time for elimination of half of the maximum plasma concentration) ranging from 11–28 h for quercetin,³⁴ 1.3–2.9 h for naringenin,³⁰ and 1.9–5.7 h for catechin.^{35,36}

The physiological activities of flavonoids are diminished by factors including lifestyle,³⁷ sex, age, race, other disease states and interactions with drugs,^{37–41} and genes which may limit their beneficial impact. These compounds would be placed to more effective use as structural guides for the design of anti-platelet therapeutic agents. At present, aspirin (acetylsalicylic acid) is the most widely used antiplatelet drug,^{42–45} but its use is associated with side effects which include stomach ulcers and bleeding^{43,46}; in addition some patients are resistant to the actions of aspirin. ADP receptor (P2Y₁₂) antagonists include the thienopyridines ticlopidine, clopidogrel, and prasugrel (CS-747; LY-640315).^{47–49} Prasugrel is increasingly replacing clopidogrel due to more potent and rapid anti-platelet effects with less inter-patient variability,^{48,49} but this drug causes bleeding complications.

Another proposed strategy for the use flavonoids as therapy could involve combining these compounds with existing anti-platelet drugs to investigate potential synergistic inhibitory effects on platelet function. A recent report showed that a mixture of resveratrol, quercetin,

and gallic acid, at relative concentrations similar to those found in most red wines, did not inhibit platelet aggregation, but that these compounds potentiated sub-inhibitory concentrations of aspirin.⁵⁰ Chocolate and cocoa were demonstrated to augment or contribute an additive inhibitory effect to anti-platelet effects of aspirin.^{51,52} Furthermore, when added to platelets that had been exposed *in vivo* to aspirin, the flavone apigenin potentiated the inhibitory effect of this drug on platelet aggregation.⁵³

Taken together, these observations between ingestion of flavonoids and CVD risk markers have encouraged research into flavonoid mechanisms of action.

1.2 Flavonoid mechanisms for inhibition of cell function

Flavonoids are well-established as antioxidants,^{54,55} but these compounds have been demonstrated to attenuate platelet function by working as pro-oxidants to enhance nitric oxide (NO).⁵⁶ These compounds may also inhibit platelet function by inhibiting reactive oxygen species (ROS)^{57,58} production, binding to cell-surface receptors,^{59–61} modifying structural proteins,^{62,63} and disrupting cell-membrane integrity.^{64,65} Exploiting flavonoid antioxidant^{54,55} and pro-oxidant^{56–58} activities and ability to bind to cell membranes^{64,65} and structural proteins^{62,63} would lead to the generation of small-molecule inhibitors which do not discriminate between target cells/tissues. It is therefore, the ability of flavonoids to inhibit kinase activity that is of particular interest, as this property suggests that these compounds may be developed further as selective therapeutic agents.

The anti-inflammatory, anti-thrombotic, and anti-proliferative properties of flavonoids are achieved through modulating the activity of kinases which drive these processes (Figure 1). Red wine polyphenolic compounds containing high levels of the flavonoids, quercetin, and catechin have been previously demonstrated to inhibit the phosphorylation of the serine/threonine kinases, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun N-terminal kinase, and protein kinase B (PKB)/Akt in vascular smooth muscle cells⁶⁶ and endothelial cells.⁶⁷ Previous reports also show that the polymeric catechin, epigallocatechin gallate (EGCG), inhibits p38 MAPK and ERK1/2 phosphorylation in platelets⁶⁸ and the tyrosine kinases (Fyn, Lyn, Btk, Syk) in mast cells.⁶⁹

Flavonoids including quercetin, apigenin, and catechin were demonstrated to inhibit the activities of tyrosine kinases (Fyn, Lyn, Syk),^{70,71} lipid kinases (phosphoinositide-3-kinase: PI3K γ),⁷¹ as well as phosphorylation of the Fc γ chain,⁷¹ phospholipase C γ 2,^{70,71} and the membrane protein, Linker and Activator of T (LAT) cells⁷¹ in platelets. The green-tea flavonoid, epigallocatechin-3-gallate and the isoflavone, genistein were shown to inhibit phosphodiesterases and Rac1 proteins which play essential roles in platelet activation, in other cell types.^{72,73} Furthermore, flavonoid-mediated increases in NO production⁵⁶ and the blocking of pathways leading to mobilization of calcium from intracellular stores^{71,74} were shown to result in the inhibition of platelet function.

The disruption of cell signalling by flavonoids may begin at the level of the plasma membrane. These compounds may decrease the fluidity of the lipid bilayer by disrupting protein–protein interactions^{64,65,75} and binding to cell surface receptors.^{59–61} Quercetin was previously demonstrated to integrate into the polar head region of dipalmitoylphosphatidylcholine liposomes (used as a phospholipid bilayer model to mimic the plasma membrane) via the A–C ring chromophore

complex⁶⁴ and to bind to thromboxane A₂ (TxA₂)⁵⁹ and adenosine diphosphate (ADP)^{60,61} receptors. This flavonol was reported to enhance the hypotonic integrity of erythrocytes after binding to their membranes.⁶⁵ Fluorescence resonance energy transfer between the tryptophan residues in erythrocyte membrane proteins and flavonoids showed that individual compounds were in close enough proximity to proteins to potentially disturb their associations.⁶⁵

Alternatively, flavonoids may be internalized by cells to gain direct access to intracellular signalling enzymes such as kinases.^{64,70,76–80} Quercetin and the methylated metabolite of this flavonol, tamarixetin, were demonstrated to be internalized by platelets⁷⁶ and megakaryocytic cells,⁷⁰ where the intrinsic fluorescent properties of these compounds allowed them to be visualized within the cytosol and associated with the platelet plasma membrane.⁷⁶

Other possible mechanisms underlying flavonoid inhibitory activities include modifications to cytoskeletal proteins including tubulin⁶² and actin.⁶³ Quercetin was suggested to inhibit microtubule assembly by masking cysteine residues on tubulin from chemical modification.⁶² Both tubulin and actin mediate platelet degranulation,^{81,82} and dense granule secretion from these cells is critically dependent on PKC Δ that mediates translocation of Syk from the cytosol to the membrane in activated platelets.⁸³ Therefore, the blocking of binding sites on the structures of cytoskeletal proteins by flavonoids may disrupt the movement of signalling proteins between intracellular compartments in stimulated platelets.

Flavonoids are therefore non-specific inhibitors, which is not entirely comparable with their potential application as clinically useful drugs. Studies investigating flavonoid inhibition of kinases indicate, however, that the activity and selectivity of these compounds is dependent on their structure, and on this basis, the search for structural elements defining the beneficial effects of these compounds was explored.

2. Understanding the link between flavonoid structure and activity

2.1 Flavonoid structure–activity relationships

The discovery that key functional groups on the flavonoid structure influence the activity of these compounds on cell function has provoked considerable interest. These compounds share a common structure, based on 2-phenyl-benzogamma-pyrane, i.e. two benzene rings (A and B rings) joined by a third pyranic (C ring) (Figure 2). The well-established free-radical scavenging abilities of flavonoids^{57,84–94} are dependent on electron-donating hydroxyl group substitutions on the aromatic B ring and heterocyclic C ring.^{86–88} The C-2–C-3 double-bond conjugated to a C-4 carbonyl group on the C ring is responsible for antioxidant activity through formation of an electron delocalization system.^{86–88}

Functional groups conjugated to the flavonoid structure through metabolism also affect the antioxidant potential of these compounds. Previous reports demonstrated that the reduction of a stable free radical, 1,1-diphenyl-2-picrylhydrazyl, was affected by the position of a glucuronide group on the flavan nucleus.⁹⁵ Quercetin-3-glucuronide prolonged the lag-time of copper-induced low-density lipoprotein oxidation less than quercetin,⁹⁶ and glucuronides of

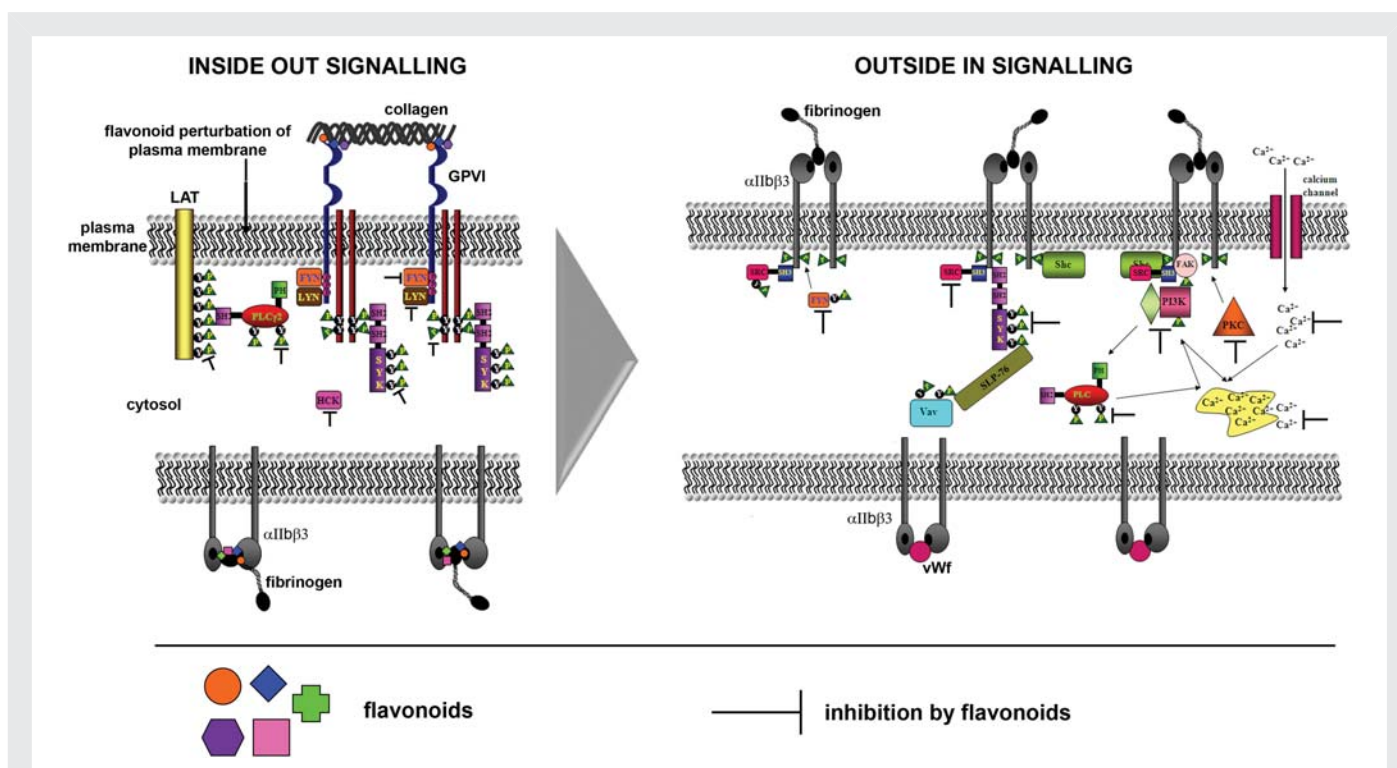


Figure 1 Inhibition of platelet signalling by flavonoids. Flavonoids inhibit tyrosine kinases (Src, Fyn, Lyn, Hck, Syk), lipid kinases (PI3K), and serine/threonine kinases (PKC) and phospholipases (PLC γ 2) involved in inside-out signalling (that initiates platelet activation) and outside-in signalling (that maintains platelet activation). These compounds also inhibit by targeting calcium signalling that is essential for platelet activation, they potentially disrupt fibrinogen and collagen binding to integrin $\alpha_{IIb}\beta_3$ and GPVI, respectively, and it is likely that they perturb the platelet plasma membrane.

catechin and epicatechin identified in the plasma of rats following ingestion of the flavan-3-ol monomers, exhibited antioxidant activities which were equivalent to that of their parent compounds.⁹⁷ Substitution of the C-3 and C-4' positions on the C and B rings (Figure 2) with a glucuronide group, respectively, was shown to diminish scavenging activity, but only a negligible decrease was observed for a glucuronide group at the C-7 position on the A ring.⁹⁵ These data are perhaps not unexpected as the hydroxyl groups (C-5 and C-7) on the A ring (Figure 2) are less important for stabilizing the flavonoid radical formed after the reduction–oxidation reaction involved during scavenging.⁸⁶

Functional groups within and on the periphery of the core flavonoid skeleton (Figure 2), including the chromenone (2H-1-benzopyran) C ring C-2–C-3 double-bond that maintains planarity, variations in the C and B ring hydroxyl group substitutions, and metabolically added sulphate and methyl groups, determine the potency of these compounds for inhibition of platelet signalling and function.^{70,98,99} The flavonol quercetin was shown to inhibit the aggregation of collagen-stimulated isolated platelets with high potency (IC_{50} : $4.9 \pm 1.0 \mu M$) attributed to the planar, C-4 carbonyl substituted C ring and B ring catechol moiety^{70,98,99} (Figure 2). The flavone apigenin inhibited platelet function with similar high potency (IC_{50} : $5.5 \pm 1.0 \mu M$) as quercetin despite the lack of C ring C-3 hydroxyl⁷⁰ (Figure 2). Low-potency inhibition of platelet function was achieved by the flavan-3-ol, catechin (IC_{50} : $545.2 \pm 74.2 \mu M$) comprising a non-planar C ring^{70,98,99} (Figure 2). Metabolic modification of quercetin with a glucuronide group significantly reduced the potency of the flavonol possibly due to reduced membrane permeability, but the

addition of a methyl or a sulphate group to quercetin allowed moderate potency inhibition of platelet function.⁷⁰

Further studies have improved our understanding of the relative importance of flavonoid functional groups. The C ring C-2–C-3 double-bond responsible for maintaining flavonoid planarity (Figure 2) was reported to be more important for high-potency inhibition of platelet function than the C ring C-4 carbonyl group, as removal of the C-4 carbonyl group on a planar C ring (cyanidin - IC_{50} : $19.2 \pm 5.3 \mu M$) resulted in approximately four-fold less potent inhibition than quercetin, whereas with a C-4 carbonyl group on a non-planar C ring (taxifolin - IC_{50} : $312.8 \pm 53.6 \mu M$), potency was reduced ~60-fold compared with quercetin.³⁵ At least two benzene rings are required for potent inhibition of platelet function. The stilbene, resveratrol comprising only two benzene rings in *cis* or *trans* conformation, inhibited platelet function with potency (IC_{50} : $5.9 \pm 1.7 \mu M$) similar to that of quercetin (unpublished data). Phenolic and benzoic acids, however, inhibited platelet function with IC_{50} ranging from 6 to 8 μM , indicating that a single-aromatic moiety (Figure 2) is not sufficient for potent inhibition (unpublished data).

Functional groups associated with potent inhibition of platelet (washed platelets) function are also attributed to potent inhibition of signalling stimulated downstream of the activatory collagen receptor, GPVI,^{58,59,70,71,98,99} ADP,^{59,98} TxA₂,^{59,98,100,101} arachidonic acid,^{53,59,98,99} and protease-activated receptors^{59,102} in these cells. The C-3 C ring hydroxyl (Figure 2) and metabolic sulphation which were not necessary for potent inhibition of platelet function were reported to be important for inhibiting platelet signalling.⁷⁰ These data are in agreement with earlier studies demonstrating that poly-

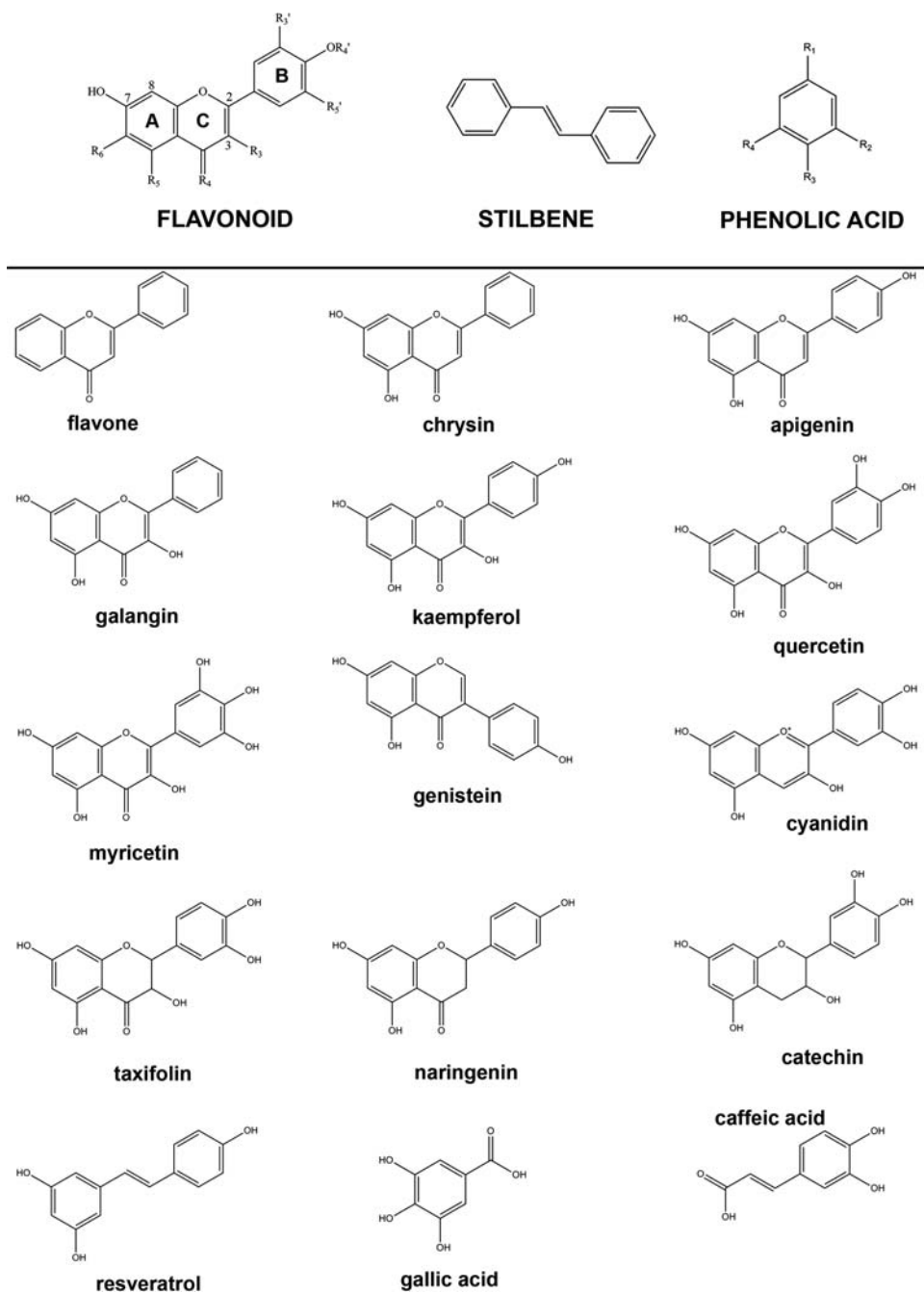


Figure 2 The structures of polyphenol compounds. Flavonoids form a major subgroup of the polyphenol family of compounds and include flavonols, flavones, flavan-3-ols, phenolic acids, isoflavones, and stilbenes. Flavones (chrysin, apigenin) are characterized by a non-hydroxylated C ring, whereas flavonol (galangin, kaempferol, quercetin, myricetin) C rings contain a C-3 hydroxyl group. Flavononols (taxifolin) are flavonols with a non-planar C ring. Flavan-3-ols (catechin) are defined by a non-planar, C-3 hydroxylated C ring that is not substituted with a C-4 carbonyl group and flavonones (naringenin) are defined by a non-planar, non-hydroxylated C ring containing a carbonyl group. Stilbenes (resveratrol) are characterized by 2 hydroxylated benzene rings in *cis* or *trans* conformations and the isoflavones (genistein) are defined by a B ring substituted to the C3 position on the C ring. Cyanidins (cyanidin) are defined by a positively charged C ring, and phenolic acids (caffeic acid and gallic acid) are benzene rings substituted with carboxyl and hydroxyl groups.

hydroxylation of flavonoids containing planar C rings were linked to potent inhibition of PI3K,^{71,103,104} PKC,^{103,104} and PIM1¹⁰⁵ activity. Further support for these findings was reported in structure-activity studies which demonstrated that removal of hydroxyl substituents from the C and B rings was attributed to decreased inhibitory potency of flavonoids for tyrosine kinase activity of the oncogene product, pp130fps.¹⁰⁶ Saturation of the C-2–C-3 double bond in addition to exclusion of C and B ring hydroxyl groups (Figure 2) were correlated with the reduced ability of flavonoids to inhibit PI3K α ¹⁰³ activity, G type (ATP and GTP-dependent) casein kinase¹⁰⁷ activity, and the catalytic activity of FOF1 rotary motors in mitochondria.¹⁰⁸

Evidence has been previously reported demonstrating that polyphenols display a degree of selectivity. An unmodified flavonol, quercetagenin, was reported to be a more selective inhibitor of the oncogene *pim-1* kinase than *pim-2* than quercetin, due to an A ring hydroxyl substitution.¹⁰⁵ Removal of the C ring C-3 and B ring C-3' hydroxyls (apigenin) and addition of a C-4' methyl group to the B ring of quercetin (Figure 2) (tamarixetin) correlated with moderate potency inhibition of Syk and low-potency inhibition of Fyn⁷⁰ involved in GPVI signalling. Therefore, apigenin and tamarixetin may preferentially inhibit Syk with greater potency than Fyn.

Inconsistencies between studies in the observed potency of flavonoids have been reported. Using quercetin as a reference compound that inhibits platelet function with high potency (low μM – nM concentrations), previous studies have shown that quercetin inhibits platelet function with intermediate (20–40 μM) potency⁵⁹ with apigenin as a more potent inhibitor of thrombus formation in whole blood than quercetin.⁵³ These differences in potency are possibly due to interactions with plasma proteins in platelet preparations.^{53,59} Certainly, inhibition of platelet function by flavonoids and their metabolites in the circulation may be influenced by several interrelated associations. Flavonoids and their biological metabolites bind to the major plasma carrier protein, human serum albumin, with differential affinities (order of affinity: tamarixetin > quercetin > naringenin > catechin)¹⁰⁹ and erythrocytes have been shown to internalize flavonols and flavones to varying degrees in a manner that correlated with structural features.^{78,80} The main objective of these types of studies is to identify structural features which underlie flavonoid potency, to indicate the manner these compounds may be translated into more selective compounds. Therefore, dietary relevant concentrations or conjugates of flavonoids are not considered. Activity in plasma is, however, an important consideration when measuring flavonoid *in vivo* effects.

With identification of discrete elements within polyphenol structures that confer potency and selectivity, a focus for beginning to construct analogues is established. The next step will be to explore molecular interactions of flavonoids with kinases to understand the manner that these compounds are positioned within catalytic sites to achieve their effects.

2.2 Structural and computational approaches to understand flavonoid inhibitory mechanisms

Flavonoids have been suggested as mapping agents to guide the development of molecular probes for enzyme/kinase catalytic sites^{106,107} which determine structural requirements for potent and potentially selective inhibition. A range of functionally diverse kinases (PI3K,^{71,103,104} PIM1, and PIM2,¹⁰⁵ myosin light chain-kinase,¹⁰⁶

casein kinase I and II,¹⁰⁶ PKC,¹⁰⁶ and PKA¹⁰⁶) with a central involvement in the growth, proliferation, and functional maintenance of nucleated cells, and key regulatory roles in signal transduction in platelets have been incorporated into initial studies of this nature.

X-ray crystallographic analyses of kinase-flavonoid co-crystals demonstrated that the flavonoid ring systems and their hydroxyl substitutions are involved profoundly in the binding of these compounds to Src-family kinases (Hck),¹¹⁰ lipid kinases (PI3K γ),¹¹¹ and serine/threonine kinases (PIM1).¹⁰⁵ A quercetin molecule bound to PI3K γ ¹¹¹ and PIM1¹⁰⁵ via an aspartic acid (Asp 964) residue involved in ring stacking with the flavan B ring (Figure 2), and the formation of hydrogen bonds with hydroxyl oxygens on the same ring. The involvement of the C ring C-3 hydroxyl in direct-binding associations was shown as the formation of a hydrogen bond between the functional group and glycine 344 within the crystal structure of Hck.¹¹¹ In contrast, van der Waals interactions occurred between this same functional group on the quercetin molecule and glutamic acid residues within PI3K γ (Glu 880)¹¹¹ and PIM1 (Glu 121)¹⁰⁵ ATP-binding sites were observed, illustrating redundancy in the interactions with the flavonol C ring hydroxyl (Figure 2) between distinct residues on structurally divergent proteins.

Hydroxyl substituents may also influence flavonoid orientations within binding sites as those varied between flavonoids with unique hydroxylation patterns as well as between different classes of proteins. Quercetin bound to the Hck substrate binding groove show a B ring that orientates towards the solvent,¹¹⁰ whereas the B ring of quercetin bound to PI3K γ is flipped inside the ATP-binding pocket.¹¹¹ Interestingly, myricetin (flavonol-B ring hydroxyl groups at C-3', C-4', and C-5') (Figure 2) co-crystallized within the PIM1 ATP-binding site¹⁰⁵ was positioned in a similar orientation as quercetin within Hck,¹¹⁰ and was bound to PI3K γ in a manner that orientated the A ring instead of the B ring towards the solvent.¹¹¹

The less effective ability of a flavonoid with a non-planar C ring (catechin) to inhibit kinase activity is supported by molecular studies. Within the ATP-binding site of DNA gyrase, EGCG [consisting of a structural homologue of catechin (epicatechin)] was orientated in a manner opposite to that of quercetin,¹¹² and a network of hydrogen bonds was formed between the flavonol and the neighbouring residues,¹¹³ but hydrogen bonds only formed between residues and the B ring of the epicatechin moiety.¹¹³

Computational tools have also been invaluable for understanding interactions between polyphenols and kinases. Analyses using the GRID algorithm (maps energy values of a functional group throughout and around kinase-binding sites)¹¹⁴ and molecular docking indicated potentially significant variations in binding modes of structurally congeneric flavonoids (quercetin, catechin, and apigenin) in Src-family kinase ATP-binding sites, which was validated with biological data (Wright and Gibbins, 2012, unpublished data). Reports of similar approaches demonstrate that myricetin, which is known to inhibit the cell cycle¹¹⁵ exerts inhibitory effects on cell proliferation by targeting Raf-1.¹¹⁶ Docking studies were performed to understand how myricetin binds to Raf1 without competing with ATP; B-Raf that is highly homologous to Raf-1 was used as the structure of Raf-1 was not available.¹¹⁶ A predicted ternary complex comprising B-Raf, ATP, and myricetin was constructed with myricetin docked to the pocket distinct from, but adjacent to, the ATP-binding site of B-Raf. The hydroxyl groups at positions 3, 5, and 7 of myricetin form hydrogen bonds with the side chains of Lys482, Thr528, and

Thr507, respectively, and hydrophobic interactions were formed with Leu504 and Val503.¹¹⁶

Other computational studies have demonstrated flavonoid interactions with housekeeping proteins. By means of molecular-docking operations, stable structures for the binding of the flavone, chrysin to calmodulin were approximated.¹¹⁷ Chrysin was situated in the region that allowed maximum contact with the side chains of the receptor and formation of proper bonds to stabilize the CaM–chrysin complex.¹¹⁷ The phosphodiesterase 4B2 catalytic site was characterized as capable of attracting a flavonoid molecule with electronegative surface charges both sterically and electrostatically.⁷³

Monomeric flavonoids were shown in molecular-docking studies to be accommodated in specific binding sites found on serine proteases involved in blood coagulation and the inflammatory response.¹¹⁸ Larger polyphenols were reported to non-specifically hinder the catalytic pocket serine proteases. The phenyl-ring in position 2 of the phenyl-benzogamma-pyranic core, the ketone carbonyl in position 4, hydroxyl groups in 3, 5, 7, and 3' (and/or 5'), and 4' position and C ring 2–3 double-bond were critical for inhibitory activity¹¹⁸ (Figure 2). A pharmacophore model classified the carbonyl group at position 4 as a H-bond acceptor towards Ser 195 acting as the H-bond donor.¹¹⁸

These structural studies prove that the tools for derivation of flavonoid-based drugs are available. At present, coherent methodology consisting of appropriate workflow modules must be developed to correctly translate these compounds into therapeutic agents.

3. The flavonoid structure as a template for drug design

The translation of flavonoids into more potent and selective small-molecules of potential therapeutic value has already begun. Quercetin is the main flavonoid used as a template for drug design. Analogues including LY294002,^{119,120} quercetin-3-*O*-amino-esters,¹²¹ and penta-*O*-substituted quercetin analogues¹¹⁹ were previously synthesized using the structure of this flavonol as a foundation (Figure 3).

Substitution of the C-8 position of the A ring with a benzene ring and omission of hydroxyl groups (LY294002) enhanced both potency and specificity for inhibition of PI3K. The small-molecule inhibitor was greater than two-fold (IC₅₀ –1.4 μM) more potent than quercetin (IC₅₀–3.8 μM) for blocking the activity of PI3K.¹²⁰ LY294002 was reported as selective for PI3K, due to complete inhibition by the compound, but PI4K, PKC, PKA, MAPK, S6 kinase, epidermal growth factor receptor tyrosine kinase activity, DAG kinase, or ATPase were not inhibited.¹²⁰ Addition of a methyl group to the C-7 position on the A ring (LY805921) did not lower inhibitory potency to as great an extent as a poly-hydroxylated and methylated heterocycle at the C ring C-3 position (LY802132) (Figure 2) or removal of the B ring catechol moiety (LY002079).¹¹⁹

Isoflavonone and isoflavanone compounds have also been used as templates for the design of more potent analogues for inhibiting interleukin activity^{122,123} (Figure 3). Planarity of the chromen-4-one ring (Figure 2), a phenolic hydroxyl at the C-4 position on the B ring

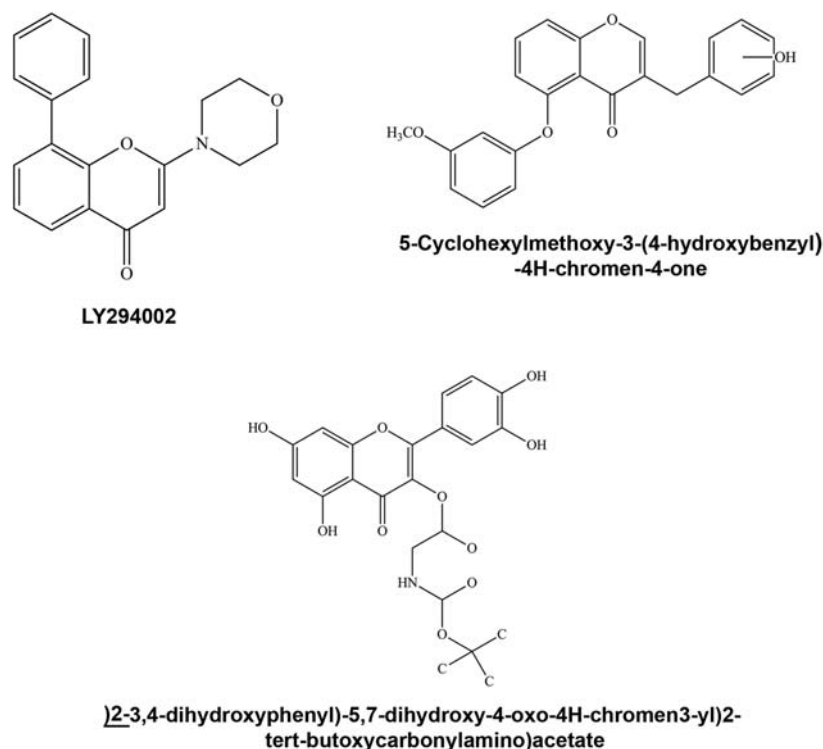


Figure 3 Flavonoid analogues. Analogues based on quercetin (LY294002 and quercetin-3-*O*-amino-esters) and the flavonoid chromone moiety (5-cyclohexylmethoxy-3-(4-hydroxybenzyl)-4H-chromen-4-one).

and a benzyloxy at the C-5 position were identified as functional groups necessary for high potency (IC₅₀ of 15.3 μM) inhibition of interleukin-5 (IL-5).¹²³ Chromone analogues synthesized based on these data included 5-cyclohexylmethoxy-3-(4-hydroxybenzyl)-4H-chromen-4-one and 5-cyclohexylmethoxy-3-(hydroxymethyl)-4H-chromen-4-one. The analogues inhibited IL-5 activity with higher potencies (IC₅₀ of 3 and 7.6 μM, respectively) than parent compounds.¹²²

Although these reported studies provide useful information, they did not explore the diversity of the flavonoid family of compounds. Strategies for comprehensive exploitation of polyphenolic compounds could involve screens of a range of compounds with clear structural differences, against key kinases involved in cell signalling.^{124–126} Previous reports employing this approach have focused on high-throughput screens of quercetin inhibition of Src and MAPK activity.¹²⁴ These approaches may involve the development of flavonoid-centred combinatorial libraries.

4. Conclusions

The scene is set for drug discovery through exploitation of dietary polyphenols. Although, long-term dietary consumption of flavonoids as a means to lower CVD risk is still an option, barriers to increasing consumption of flavonoid rich foods, particularly fruits and vegetables, and possible lack of specificity could have a significant impact on the efficacy of this strategy for risk reduction.

To explore the potential of these compounds as templates applied to the construction of potent, selective small molecule inhibitors, thorough screenings of their structural interactions with molecular targets are necessary. This approach can be accelerated through the use of computational tools together with structural assays, before follow-up investigations involving biological validation of actual/approximated interactions between kinases and polyphenols. This platform of methodologies may include modelled kinases as well as those with solved structures, and therefore allow the inclusion of as broad a range of kinases as possible. Furthermore, this rational screening strategy performed to understand features on the flavonoid structure which confer selectivity may involve recombinant kinase libraries and model cell systems (e.g. platelets).

The development of a new generation of flavonoid-based inhibitory agents may overcome the common problem encountered with the small therapeutic window of or lack of efficacy of existing anti-platelet drugs.

Conflict of interest: none declared.

Funding

Research in the authors' laboratory is supported by grants from the Medical Research Council, British Heart Foundation, Wellcome Trust, and the Biotechnology and Biological Sciences Research Council.

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